ELSEVIER

Journal of Chromatography A, 955 (2002) 87–93

JOURNAL OF CHROMATOGRAPHY A

www.elsevier.com/locate/chroma

Screening of *Catharanthus roseus* secondary metabolites by high-performance liquid chromatography

C. Tikhomiroff, M. Jolicoeur*

Bio-P² Research Unit, Department of Chemical Engineering, École Polytechnique de Montréal, P.O. Box 6079, Centre-ville Station, Montréal, Québec H3C 3A7, Canada

Received 3 September 2001; received in revised form 25 February 2002; accepted 25 February 2002

Abstract

Two direct HPLC analytical methods for the screening of the major indole alkaloids of *Catharanthus roseus* hairy roots and their iridoid precursors have been developed. Photodiode array and fluorescence detection were performed. The separation was achieved on a reversed-phase C_{18} column. The first method allowed the separation of catharanthine, serpentine, tabersonine, vindoline, vinblastine, and vincristine in 20 min. Ajmalicine, tryptophan, tryptamine and secologanine were separated using the second method in 13 min. The identification of the compounds was based on the retention time and the comparison of UV spectra with those of authentic standards. A simplified alkaloid extraction method was developed in order to accelerate sample preparation. The assays were successfully used to quantify major compounds of the secondary metabolism of hairy root cultures of *C. roseus*, thus providing a reliable tool for rapid screening of *C. roseus* secondary metabolite samples. In these cultures, ajmalicine, serpentine, catharanthine, tabersonine, and tryptamine were detected, but tryptophan, vindoline, vinblastine and vincristine were not.

Keywords: *Catharanthus roseus*; Plant materials; Terpenoids; Indoles; Alkaloids; Iridoids

1. Introduction

The production of secondary metabolites by plant cells and tissues has become an active field of study because of its potential as a source of valuable pharmaceutical compounds. In this context, in vitro cultures of plant cells or tissues look promising for the large scale production of secondary metabolites. Indeed, such cultures are not exposed to disease and pests, and seem not to be subject to seasonal and somatic variations. Moreover, some new compounds have been discovered in these cultures. *Catharanthus roseus* produces widely used alkaloids such as the anticancer drugs vinblastine and vincristine, as well as the antihypertensive compounds ajmalicine and serpentine. Catharanthine, tabersonine, lochnericine and horhammericine are other indole alkaloids found in *C. roseus*. *Agrobacterium rhizogenes* transformed hairy root cultures have been successfully used to produce some of these alkaloids [1,2]. One of our research interests is the study of *C. roseus* hairy roots secondary metabolism under different environmental conditions, such as elicitation, and nutritional states. The secondary metabolism pathway of indole alkaloids in *C. roseus* has already been documented [3,4] and is quite complex (Fig. 1). Tryptophan is

*Corresponding author. Tel.: +1-514-340-4711x4525; fax: +1-514-340-4159.

E-mail address: mario.jolicoeur@polymtl.ca (M. Jolicoeur).

0021-9673/02/$ – see front matter
PII: S0021-9673(02)00204-2

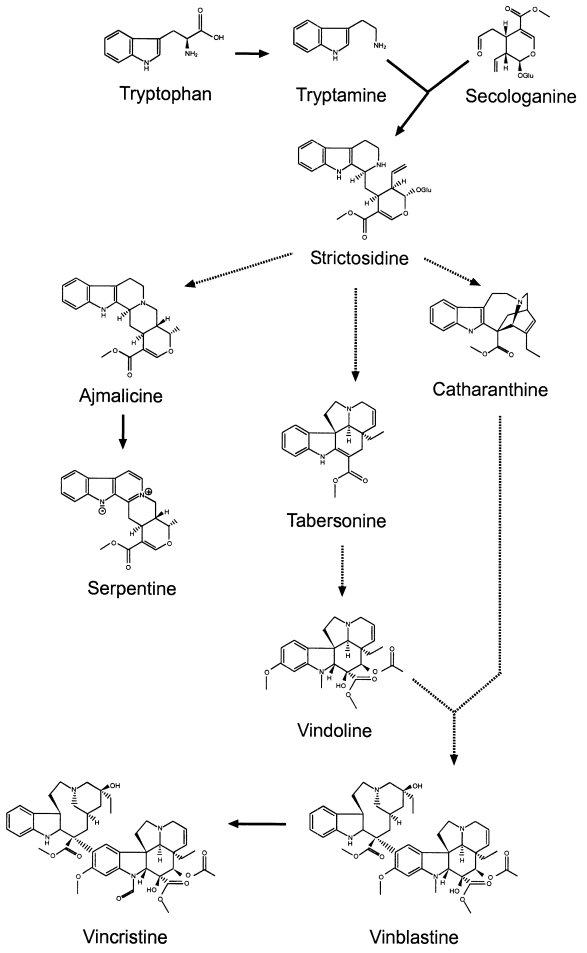

Fig. 1. Metabolic pathway of biosynthesis of indole alkaloids in *C. roseus* (adapted from Refs. [3,4]). Dashed arrows indicate multi-step reactions.

converted to tryptamine. Tryptamine is condensed with secologanine to yield strictosidine, the common precursor of all indole alkaloids, divided into three branches. The production of indole alkaloids is strongly regulated by environmental conditions and cell growth status [5]. A better understanding of the metabolic fluxes' regulation can be obtained using metabolic flux analysis (MFA) [6], which requires determination of the biosynthetic reaction rates. This method also allows the validation of new metabolic fluxes. Nevertheless, it requires a large number of measurements. Thus, only a rapid quantification of indole alkaloids makes it feasible for studying *C. roseus* secondary metabolism.

In order to measure the intracellular concentration of alkaloids, one has to extract, purify and analyze the alkaloids from plant cells or hairy roots [7]. The extraction from lyophilized plant is performed with methanol, ethyl acetate or chloroform. Soxhlet extraction can be used to dissolve the secondary metabolites in an organic phase [1]. However, Soxhlet extraction may alter the chemical nature of the compounds under study and this procedure is time-consuming. Sonication of the sample in methanol may be used instead of the Soxhlet extraction [7]. Crude plant extracts often need to be concentrated and fractioned in order to remove lipids, proteins, pigments and other plant cell compounds. Once the samples have been purified, the separation and quantification is performed. Thin-layer chromatography (TLC) and colorimetry have been used to isolate and quantify indole alkaloids of *C. roseus* [7–10]. However, this method is not suitable for routine analysis when numerous samples are involved. Moreover, crude plant extracts often contain many alkaloids and TLC is not always able to separate them adequately.

High-performance liquid chromatography (HPLC) systems equipped with an autosampler provide a powerful tool to analyze numerous samples. The separation of indole alkaloids is based on reversed-phase chromatography using C_{18} as a stationary phase. Mobile phases usually consist of a mixture of a buffer solution such as *n*-heptanesulfonic acid [1,7], diammonium phosphate [11], or ammonium acetate supplemented with triethylamine [12] and an organic phase (such as methanol or acetonitrile). Detection is performed using a UV detector at fixed wavelength [1,12] or a fluorescence detector [11]. All these methods allowed quantification of most of the indole alkaloids of *C. roseus*. However, to study the overall secondary metabolism of *C. roseus*, the iridoid precursors, such as secologanine, and the indole precursors, such as tryptophan and tryptamine also need to be quantified. Recently, a novel HPLC method was proposed to quantify these compounds in a crude extract of *C. roseus* [13]. The indole alkaloid precursors were extracted in methanol. This result suggested that one methanolic crude plant extract could be used to analyze both indole alkaloids and their precursors. Nevertheless, the overall procedure for quantification of indole alkaloids

remains quite complicated if extraction, concentration, purification and analysis all have to be conducted.

This work presents the development of two HPLC methods that allow the quantification of indole alkaloids and their precursors in two distinct runs of 30 min and 20 min. These methods were optimized with a simplified sample preparation consisting of methanol extraction from lyophilized biomass without purification and concentration in order to significantly shorten sample preparation. The originality of the developed methods lies in the simplification of the entire analysis procedure of known terpenoid indole alkaloids in *C. roseus* hairy roots.. Thus, it renders feasible the evaluation of metabolic fluxes during a culture of *C. roseus* hairy roots.

2. Experimental

2.1. Chemicals

Small amounts of ajmalicine, serpentine, catharanthine, tabersonine and vindoline were kindly provided by Dr. Archambault (UQTR, Quebec, Canada). Secologanine (99% of purity) was purchased from Phytoconsult (Gorlaeus Labs., Leiden, The Netherlands). Tryptamine, tryptophan, vincristine and vinblastine (98% of purity) were purchased from Sigma–Aldrich (Oakville, Canada).

2.2. Apparatus and chromatic conditions

The HPLC analysis was performed using a Beckman Coulter pump module 126, a Beckman Coulter auto-sampler model 508, a Beckman Coulter photodiode array detection (DAD) system 168 and a Jasco model 821-FP fluorescence detector. A Zorbax Eclipse XDB-C_{18} 250 mm×4.6 mm column (Hewlett-Packard, Missisauga, Canada), coupled with an Upchurch Scientific 1 cm×4.3 mm ODS guard column (Upchurch Scientific, Concord, Canada), was used at a column temperature of 35 °C. The injection volume was 10 μl.

Method I was used for the quantification of catharanthine, serpentine, tabersonine, vindoline, vinblastine and vincristine. The mobile phase consisted of a mixture of 5 m*M* Na_2HPO_4 (pH adjusted to 6 with H_3PO_4) (solvent A) and acetonitrile (solvent B). Flow-rate was 2.0 ml/min. The eluent profile (volume of solvent A/volume of solvent B) was: 0–20 min, linear gradient from 80:20 to 20:80; 20–25 min, isocratic elution with 20:80 (v/v) (column rinsing); 25–30 min, isocratic elution with 80:20 (v/v) (column equilibration).

Method II was used for the quantification of tryptophan, tryptamine, secologanine and ajmalicine. The mobile phase consisted of a mixture of 100 m*M* H_3PO_4–acetonitrile (85:15, v/v) . Flow-rate was 1.8 ml/min. The column was rinsed with a 15:85 (v/v) mobile phase for 5 min and equilibrated for 3 min.

2.3. Standard solutions

Individual stock solutions of ajmalicine, catharanthine, secologanine, tabersonine, tryptamine, vindoline, vincristine and vinblastine were prepared at a concentration of ~5 mg/ml in methanol. Individual stock solutions of tryptophan and serpentine were prepared at a concentration of ~5 mg/ml in water–methanol (50:50, v/v). These stock solutions were stored at −20 °C. The quantification was performed using six levels of external standards. Level 6 consisted of a dilution (1:46, v/v) of stock solution in methanol. Levels 5 to 1 were obtained by dilution of level 6 by 2, 4, 8, 16 and 32. The ranges obtained were 2–4 μg/ml to 64–128 μg/ml depending on the concentration of each alkaloids' stock solution.

2.4. Sample preparation

Approximately 200 mg of fresh hairy roots were lyophilized overnight (~10 h). The dry roots were weighed, crushed in a tissue grinder (VWR Canlab, Montréal, Canada) and extracted at room temperature in 1 ml of MeOH for 60 min in a sonicating bath. The extract was centrifuged at 15,000 *g* for 5 min at room temperature and the supernatant was filtered through a PTFE 0.45 μm filter into an amber glass HPLC vial prior to HPLC analysis.

2.5. Peak identification, peak purity, limits of detection and extraction yield

Alkaloids and their precursors were extracted from lyophilized hairy roots of *C. roseus*. Samples were

analyzed as is or spiked with known amounts of standards (~4 mg per g dry mass of hairy roots).

Identification of alkaloids from the crude plant extract was established by comparison of the UV spectra and retention time with those of authentic standards. The peaks' purity determination was based on the ratio of absorbance at 220 nm to absorbance at 280 nm compared to that of standards [14,15]. The limit of detection was set where the ratio of the standard's peak area to noise was greater than three.

The extraction yield was evaluated using the following formula:

$$\text{extraction yield} = (C_{i+a} - C_i)/C_w$$

where C_{i+a} is the HPLC measured concentration (mg/g dry mass hairy root) of metabolite in the spiked sample, C_i the HPLC measured concentration (mg/g dry mass hairy root) of metabolite in the non-spiked sample and C_w is the concentration (mg/g dry mass hairy root) of weighted metabolite added to spiked samples. For all compounds, both UV spectra and retention times of spiked samples were identical to those of the standards.

2.6. Hairy root cultures of C. roseus

C. roseus L.G. Don hairy roots were established as already described [1]. Root cultures were grown in Petri dishes on minimum medium [16] supplemented with 3% (w/v) sucrose, as well as a 10-fold KH_2PO_4 (8.1 m*M* NO_3 final) and a threefold $Ca(NO_3)_2$ (3 m*M* NO_3 final) concentration to obtain a specific nutritional status ideal for our research (experiments) [17]. The cultures were elicited using 25 mg/l jasmonic acid [18] during the exponential growth phase and harvested 3 days later for quantification of indole alkaloids and precursors.

3. Results and discussion

3.1. Separation of standards

All studied alkaloids and iridoid precursors were first characterized individually to record their UV spectrum. All mobile phases consisted of potassium phosphate buffer (5–100 m*M*) with a pH of 2, 4, 6 or 8 and an organic solvent (methanol, acetonitrile and acetonitrile + 0.05–0.1% (v/v) methanol). Optimal chromatographic conditions were obtained after testing different mobile phases with a reversed-phase C_{18} column. Isocratic elutions resulted in a poor separation of alkaloids unless very long methods were used. Linear gradient elutions were also investigated and resulted in good separation of serpentine, catharanthine, vindoline, vinblastine, vincristine and tabersonine. However, the separation of ajmalicine, serpentine and catharanthine was shown to be pH dependent. Moreover, alkaloid precursors were not separated. Thus, the use of two methods with distinct eluents became a necessity to separate all studied compounds.

Method I provided separation of serpentine, catharanthine, vindoline, vinblastine, vincristine and tabersonine (Fig. 2A). The mobile phase was composed of a mixture of phosphate buffer (pH 6)–acetonitrile with the following elution profile: linear gradient from 80:20 (v/v) to 20:80 (v/v) in 20 min followed by an isocratic elution at 20:80 for 5 min at a flow-rate of 2 ml/min. Accuracy of the pH was critical to achieve the separation. Method II was inspired by the work of Dagnino et al. [13], and was used to separate tryptophan, tryptamine, sec-

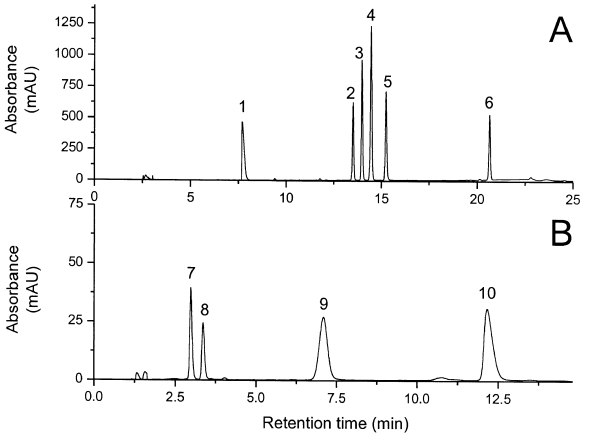

Fig. 2. HPLC–DAD chromatogram of pure standards with injection volume of 10 μl. (A) Method I, 210 nm. 1, Serpentine; 2, vincristine; 3, vindoline; 4, catharanthine; 5, vinblastine; 6, tabersonine. Mobile phase: linear gradient from 80:20 to 20:80 in 20 min of 5 m*M* phosphate buffer (pH 6)–acetonitrile. (B) Method II, 238 nm. 7, Tryptophan; 8, tryptamine; 9, secologanine; 10, ajmalicine. Mobile phase: isocratic elution, 100 m*M* phosphate buffer (pH 2)–acetonitrile (85:15).

ologanine and ajmalicine in less than 13 min (Fig. 2B). An isocratic elution composed of phosphoric acid (100 m*M*)–acetonitrile (85:15, v/v) was run at a rate of 1.8 ml/min. The high concentration in phosphate buffer was needed to minimize peak tailing. Both methods demonstrated an efficient separation of all standards (Fig. 2).

The detection of alkaloids was done by DAD using the 3D mode, allowing collection of UV spectra of the compounds from 210 to 360 nm by steps of 2 nm in real time and checking the peak purity. Quantification of serpentine, catharanthine, vindoline, vincristine, vinblastine, tabersonine and secologanine were performed at a wavelength near their maximum UV absorbance wavelength. Tryptophan, tryptamine and ajmalicine were quantified with fluorescence response at 370 nm for an excitation wavelength of 270 nm, although they could also be detected and quantified by DAD. Fluorescence was used to get a higher sensibility of detection. Table 1 provides retention time, DAD wavelength, range of quantification, and limit of detection for all standards. The injection volume was 10 μl for both methods.

3.2. Analysis of a crude plant extract

A methanolic extract of *C. roseus* hairy roots was injected to evaluate the separation efficiency of the alkaloids (Fig. 3). With method I, an unknown compound co-eluted with serpentine that has at least two maximum absorbance wavelengths (250 and 306 nm). The unknown compound did not interfere with serpentine at 306 nm, so it was still possible to quantify serpentine at this wavelength. Catharanthine and tabersonine were detected as pure compounds. As reported previously [19], vincristine, vindoline and vinblastine were not detected in hairy root cultures of *C. roseus* of the present study. Method II allowed the separation of tryptamine, secologanine and ajmalicine from the same crude extract as pure

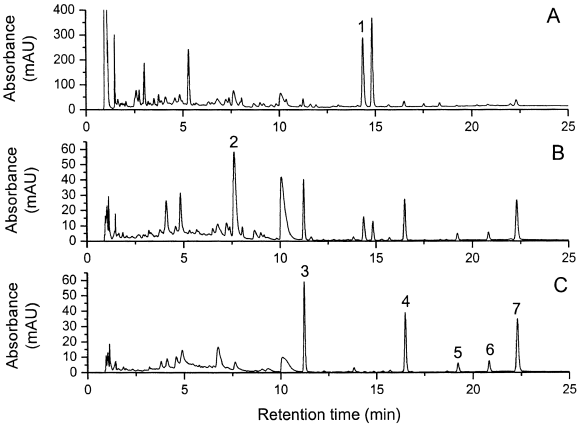

Fig. 3. Method I DAD–HPLC chromatograms of *C. roseus* hairy root extract with injection volume of 10 μl. 1, Catharanthine; 2, serpentine; 3, unknown T1; 4, unknown T2; 5, unknown T3; 6, tabersonine; 7, unknown T4. Mobile phase: linear gradient from 80:20 to 20:80 in 20 min of 5 m*M* phosphate buffer (pH 6)–acetonitrile. Chromatograms at 220 nm (A), 306 nm (B) and 330 nm (C).

Table 1
Detection parameters of indole alkaloids and precursors (methods I and II)

Metabolite	Retention time min	Wavelength nm	Test range μg/ml	Limit of detection μg/ml	Extraction yield (%)[a]
Serpentine	7.8	306	3.0–96.1	0.47	32.4±4.3
Vincristine	13.1	220	2.68–85.8	0.42	43.2±9.9
Vindoline	13.5	220	3.05–97.6	0.52	39.2±3.2
Catharanthine	14.5	220	2.84–90.8	0.49	24.5±1.5
Vinblastine	15.2	220	3.7–118	0.61	88.2±16.2
Tabersonine	20.8	330	2.72–87.1	0.093	34.5±1.2
Tryptophan	3.2	270/370[b]	1.77–56.6	0.043	54.8±8.4
Tryptamine	3.5	270/370[b]	1.52–48.5	0.034	85.4±23.4
Secologanine	7.2	238	2.73–87.4	0.75	110.8±5.3
Ajmalicine	12.2	270/370[b]	3.63–116	0.97	98.3±7.81

[a] Intervals are standard deviations ($n=3$).
[b] Fluorescence detection at excitation/emission wavelengths.

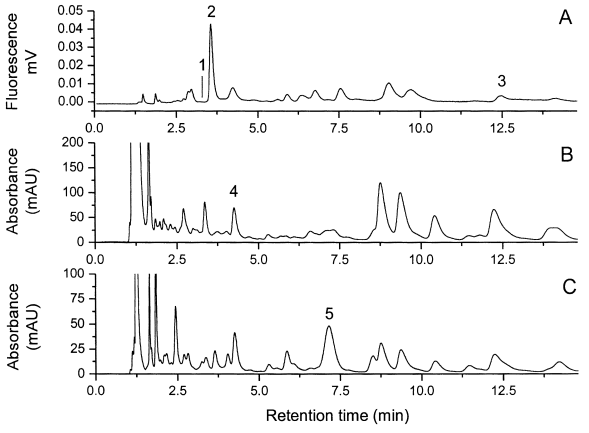

Fig. 4. Method II HPLC chromatograms of a *C. roseus* hairy root extract with injection volume of 10 μl. 1, Tryptophan; 2, tryptamine; 3, ajmalicine; 4, unknown T5; 5, secologanine. Mobile phase: isocratic elution, 100 m*M* phosphate buffer (pH 2)–acetonitrile (85:15). Fluorescence response at 370 nm for an excitation wavelength of 270 nm (A). UV absorbance at 210 nm (B) and 238 nm (C).

compounds (Fig. 4). However, only traces of tryptophan were detected in all assays and no other compound was detected at the tryptophan retention time. The calibration curves exhibited linear regression ($r > 0.997$) for both HPLC methods.

To verify the validity of our procedure, the extraction yield was measured for each studied compound. The extraction yield was shown to be very dependent on the compound (Table 1). Ajmalicine and secologanine were completely extracted from the hairy roots whereas catharanthine, serpentine, tabersonine, vindoline and vincristine were only partially extracted (Table 1). Although the proposed extraction method did not result in 100% recovery for all studied compounds, it was appropriate for the screening of indole alkaloids and its precursors in hairy roots of *C. roseus*. Internal standards should be used when high accuracy is required.

The chromatograms of crude extract of *C. roseus* exhibited five pure unknown compounds (T1–T4, Fig. 3C; T5, Fig. 4B) whose UV spectra share high similarities to the tabersonine UV spectrum but have different retention times: 11.2 min (3), 16.5 min (4), 19.2 min (5), 22.3 min (7) (Fig. 3C), and 4.2 min (4) (Fig. 4B). NMR and MS studies will be done to determine the structure of these compounds. However, their UV spectrum suggests they might be related to the secondary metabolism of *C. roseus*. This is interesting, since it demonstrates that both HPLC methods have the potential of good separation of other indole alkaloids or precursors.

4. Conclusion

Two effective reversed-phase HPLC methods were developed that allowed the separation of eight *C. roseus* indole alkaloids, as well as three iridoids and indole precursors in *C. roseus* crude extracts. Catharanthine, serpentine, tabersonine, ajmalicine, secologanine and tryptamine were successfully quantified in elicited hairy root cultures, whereas vindoline, vincristine, vinblastine and tryptophan were not detected. Efficient separation of alkaloids and precursors was achieved in the crude plant extracts despite a simplified extraction procedure. The rapid quantification of many alkaloids and precursors will enable future studies on the metabolic fluxes of terpenoid indole alkaloids of *C. roseus* hairy roots.

Acknowledgements

C.T. has a fellowship from the Fondation de l'École Polytechnique (France). The HPLC system was purchased with a grant from the National Sciences and Engineering Research Council of Canada (NSERC). The Fonds pour la Formation de Chercheurs et l'aide à la Recherche (FCAR) and the NSERC funded this research project. The authors wish to thank M. Klvana and N. Chauret for reviewing this document.

References

[1] R. Bhadra, S. Vani, J.V. Shanks, Biotechnol. Bioeng. 41 (1993) 581.
[2] A.J. Parr, A.C.J. Peerless, J.D. Hamill, N.J. Walton, R.J. Robins, M.J.C. Rhodes, Plant Cell Rep. 7 (1988) 309.
[3] J.A. Morgan, J.V. Shanks, Phytochemistry 51 (1999) 61.
[4] V. De Luca, P. Laflamme, Curr. Opin. Plant Biol. 4 (2001) 225.
[5] B. St-Pierre, F.A. Vazquez-Flota, V. De Luca, Plant Cell 11 (1999) 887.

[6] G.N. Stephanopoulos, A.A. Aristidou, J. Nielsen, Metabolic Engineering, Academic Press, San Diego, CA, 1998.
[7] P. Morris, A.H. Scragg, N.J. Smart, A. Stafford, in: Plant Cell Culture, A Practical Approach, IRL Press, Oxford, 1985, p. 127, Chapter 7.
[8] F. Vazquez-Flota, O. Moreno-Valenzuela, J. Miranda-Ham, J. Coello-Coello, V.M. Loyola-Vargas, Plant Cell Tissue Organ Cult. 38 (1994) 273.
[9] M. Montforte-Gonzalez, T. Ayora-Talavera, I.E. Maldonado-Mendoza, V.M. Loyola-Vargas, Phytochem. Anal. 3 (1992) 117.
[10] N.R. Farnsworth, R.N. Blomster, D. Damratoski, W.A. Meer, L.V. Camparato, Lloydia 27 (1964) 302.
[11] J.-P. Renaudin, Physiol. Vég. 23 (1985) 381.
[12] L. Toivonen, M. Ojala, V. Kauppinen, Biotechnol. Bioeng. 37 (1991) 673.
[13] D. Dagnino, J. Schripsema, R. Verpoorte, Planta Med. 62 (1995) 278.
[14] H. Cheng, R.R. Gadde, J. Chromatogr. Sci. 23 (1985) 227.
[15] A.F. Fell, H.P. Scott, J. Chromatogr. A 273 (1983) 3.
[16] G. Bécard, J.A. Fortin, New Phytol. 108 (1988) 211.
[17] C. Tikhomiroff, M.Sc.A. Thesis, École Polytechnique de Montréal, 2001.
[18] S.K. Rijhwani, J.V. Shanks, Biotechnol. Prog. 14 (1998) 442.
[19] P.R.H. Moreno, R. van der Heijden, R. Verpoorte, Plant Cell Tissue Organ Cult. 18 (1995) 1.